VACCINE AGAINST LIPOPOLYSACCHARIDE CORE

Cross-reference to related application

This application is a conversion of our prior U.S.
5 provisional application, U.S. 60/046,680, filed May 16,
1997, which is hereby incorporated by reference.

Field of the Invention

This invention is in the general field of reducing
the adverse effects of endotoxin from Gram-negative
10 bacteria.

Background of the Invention

Endotoxin (also called lipopolysaccharide [LPS])
is thought to exert many of its toxic effects following
its entry into the bloodstream. The presence of
15 endotoxin in the blood, endotoxemia, can occur in various
situations, e.g., during periods of stress. For example,
endotoxemia can occur in patients undergoing certain
types of surgery, anti-cancer chemotherapy, radiation
therapy, and immunosuppressive treatment, and it can also
20 occur in patients suffering from various trauma, burns,
or wounds. It occurs as well in military, police, and
fire-fighting personnel as well as in endurance athletes,
horses, and in livestock. It can also occur after
immunosuppressive treatment, and in patients with sepsis
25 or septic shock as well as in those suffering from stress
or trauma as discussed above.

One way that endotoxin may reach the blood is from
the patient's intestine because the intestine loses its
ability to contain LPS during periods of infection,
30 stress, or trauma. Normally, intestinal flora contain a
large amount of endotoxin from Gram-negative
microorganisms. It is estimated that the average human
colon contains 25 billion nanograms of endotoxin, which
is an enormous quantity when one considers that endotoxin
35 concentrations on the order of 10^2 are toxic to humans.

- 2 -

The leakage of live bacterial cells into the bloodstream can result in infection as the bacteria multiply. Many of the bacteria in the intestine are dead, and endotoxin contained within cell membrane
5 fragments of dead bacteria can also enter the bloodstream. In this case infection per se does not develop. Instead, endotoxin from dead bacteria in the blood is thought to initiate a systemic inflammatory response by activating macrophages which release tumor
10 necrosis factor and various interleukins. Endotoxin exposure and the resulting systemic inflammatory response can cause damage to body organs, including the lungs, kidneys, heart, blood vessels, gastrointestinal tract, blood/coagulation system, and nervous system. This
15 proinflammatory response can be severe, causing organs to fail, sometimes resulting in death.

LPS is thought to be a major causative agent of septic shock. It is increasingly recognized that less severe forms of this systemic inflammation cause organ
20 dysfunction as opposed to organ failure. In its mildest form, endotoxemia can cause fever, nausea, and malaise, common symptoms of patients following surgery or patients who are hospitalized for other reasons, and the symptoms even occur, for example in athletes following strenuous
25 activity.

Greater exposure of the host to endotoxin or a greater susceptibility to its effects can result in a larger inflammatory response. For example, many post surgical patients develop pulmonary dysfunction requiring
30 supplemental oxygen. They may also develop hematologic or renal complications. These complications often do not lead to death but instead cause suffering and increase hospital length of stay and thus health care costs. It is estimated that at least 10% of the 28 million United
35 States surgical patients may develop systemic

- 3 -

inflammation and possible complications as a result of
exposure to endotoxin from Gram-negative microorganisms.
See generally, Bennett-Guerrero et al., Crit Care Med,
25:A112, 1997 and Bennett-Guerrero et al., J. Am. Med.
5 Ass. 277:646-650, 1997 discussing certain types of
surgical patients at risk.

     Among the bacteria and their respective endotoxins
which are thought to commonly cause complications are
Escherichia coli, Klebsiella pneumoniae, Pseudomonas
10 *aeuruginosa, Proteus spp., Enterobacter spp., Salmonella*
spp., Serratia spp, and Shigella spp. These bacteria are
Gram-negative bacteria, a class characterized by a
specific type of outer membrane which compromises a
lipopolysaccharide (LPS) as a major constituent.
15 Although the LPS constituent varies from one bacterial
species to another, it may be generally described with
reference to Fig. 1 as consisting of three structural
regions: a) Lipid A; b) core; and c) O-polysaccharide
outer region. The lipid region of Lipid A is embedded in
20 the outer leaflet of the outer membrane. The
oligosaccharide core region is positioned between Lipid A
and the O-polysaccharide outer region. Lipid A has the
same basic structure in practically all gram negative
bacteria and is the main endotoxic determinant. The LPS
25 core region shows a high degree of similarity among
bacterial genera. It usually consists of a limited
number of sugars. For example, the inner core region is
constituted of heptose and 3-deoxy-D-*manno*-2-octulosonate
(KDO) residues, while the outer core region comprises
30 galactose, glucose, or N-acetyl-D-glucosamine residues
displayed in various manners depending upon the strain.
The O-polysaccharide outer region (also called O-specific
antigen or O-specific side chain) is highly variable and
is composed of one or more oligosaccharide repeating
35 units characteristic of the serotype.

- 4 -

The presence of the O-polysaccharide side chain confers a smooth aspect to a culture of a wild type bacterium, and, for this reason, wild type bacteria with polysaccharide side chain are usually referred to as

5   smooth bacteria in contrast with mutant cultures which show a rough aspect because they lack the O-polysaccharide side chain and (in some cases) part of the core region. For example, the different chemotypes of rough mutants from Salmonella are conventionally

10   designated by the terms Ra, Rb, Rc, Rd, and Re.

As seen from Fig. 2, the LPS of each type comprises the lipid A structure. The Ra chemotype is characterized by a complete core region, the Rb chemotype is characterized by the absence of N-acetyl-D-glucosamine

15   residues, the Rc chemotype is characterized by the absence of N-acetyl-D-glucosamine and galactose residues, the Rd chemotype is characterized by the absence of any residues constituting the outer core, and the Re chemotype is characterized by the sole KDO region

20   attached to lipid A.

Fig. 3 is a diagrammatic representation of the five known complete core chemotypes of E. coli as well as the one known complete core chemotype of all Salmonella species.

25   Not all LPS molecules on the surface of a given cell or in a homogeneous population of cells have the same number of oligosaccharide side chains. For example, a single cell from a population of smooth strain bacteria may include some rough forms of LPS, i.e., LPS that is

30   not substituted with any polysaccharide side chains.

Various treatments for the toxic effect of LPS have been proposed or tried. One of a mammal's defenses against endotoxemia is the presence of antibodies in the blood which can bind to and neutralize blood borne

35   endotoxin, and immunologic methods have been proposed as

- 5 -

an alternative or additional treatment to antibiotic
therapy to prevent or control such infections or to
reduce the toxic effect of endotoxin. For example,
conventional polyclonal antisera and hyperimmune sera
5 have been used in an attempt to bolster the native
defenses of patients against the adverse effects of
bacteria, presumably by enhancing opsonization and
phagocytosis of the bacterial cells or by neutralization
of the biological activity of LPS. However, the
10 effectiveness of the antisera varies greatly depending
upon a large number of factors including, for example,
the composition and titer of the specific antibodies,
which cannot be easily standardized. The use of these
antisera may also carry a risk of transmission of viral
15 infectious diseases.

        Patients or potential donors of hyperimmune sera
have been vaccinated (i.e. actively immunized) with
various immunogens in an attempt to stimulate the host
synthesis of cross-reactive anti-endotoxin antibodies.
20 Various vaccine compositions and methods of immunization
have been studied over the last two decades. See, e.g.,
Bhattacharjee A, WO 95/29662; McCabe WR, J Infec Dis
1988; 158:291; Greisman SE, Proc Soc Exp Bio Med 1978;
158:482; Goto M, Res Comm Chem Path Pharm 1992; 76:249;
25 DeMaria A, J Infec Dis 1988; 158:301; Baumgartner JD, J
Infec Dis 1991; 163:769; Cross A, J Infec Dis 1994;
170:834; Cryz SJ, US 4,755,381; Miler JM, J Med Microbiol
1977; 10:19; Ashton, FE, Microb Pathog 1989; 6:455;
Dorner F, US 4,946,677; Cryz SJ, US 4,771,127; Collins
30 MS, US 4,693,891; Pier GB, US 4,285,936; Cryz SJ, J Infec
Dis 1991; 163:1040; Cryz SJ, J Clin Invest 1987; 80:51;
PCT WO92/06709; US 5,641,492.

        Vaccines should avoid the common side effects --
fever, malaise, and other forms of toxicity -- in the
35 animals and humans receiving them, as reported, for

- 6 -

example, in (Ziegler et al., New Eng. J. Med., 307:1225
(1982); DeMaria, Infec. Dis. 158:301 (1988). These side
effects may be exacerbated in high risk individuals, such
as pre-surgical elderly and sick patients with multiple
5   medical problems, an important target population for the
vaccine.

A study has been published in which the
concentration of anti-endotoxin-core antibodies was
measured in 301 patients prior to cardiac surgery and the
10   relationship to postoperative outcome tested. Bennett-
Guerrero et al. J. Am. Med. Ass. 277:646 (1997). These
anti-endotoxin-core antibodies were measured using an
ELISA which allegedly detects antibodies to the core of
LPS. This study found that patients with a higher level
15   of core-specific antibodies were less likely to die or
have a prolonged hospital length of stay associated with
complications potentially attributable to endotoxemia.
This publication did not describe a method for
controlling or treating endotoxemia in these patients.
20   Regarding the toxicity of LPS, there is some
evidence that the toxicity of certain types of lipid A or
LPS can be reduced in several ways.

Liposomes have been suggested as carriers for
lipid A containing agents. For example, incorporation of
25   LPS into liposomes was shown to reduce the toxicity of
LPS from Neisseria meningitidis in an attempt to create a
vaccine specific for _N. meningitides_ LPS. Petrov et al.,
Infect. Immunity 60:3897 (1992). Other prior art showed
that the incorporation of a chemically altered form of
30   lipid A called monophosphoryl lipid A (MPLA) into
liposomes resulted in reduced toxicity of the MPLA
component. Richards et al. Vaccine 7:506 (1989). It
appeared as if incorporating the MPLA and malaria
immunogen into the liposomes resulted in an adjuvant
35   effect, that is, incorporation into liposomes increased

- 7 -

the immunogenicity of the malaria immunogen component of this anti-malaria vaccine.

Methods to detoxify the lipid A component of LPS have also been previously described. Bhattacharjee et
5   al. J. Infec. Dis. 173:1157 (1996).

Genetic alteration of bacterial stains has been reported in which the resulting bacteria contain a LPS whose lipid A component causes less biological toxicity. Somerville et al., J. Clin. Invest. 97:359 (1996).

10   Summary of the Invention
Vaccination (active immunization) with complete core rough LPS antigen particularly from E. coli K12 provides both strain-specific protection and cross-core protection without unacceptable toxicity or other side
15   effects. An antigen is considered a complete-core, rough LPS in that it includes, at a minimum, Lipid A, heptose and 3-deoxy-D-manno-2-octulosonate (KDO) residues, as well as the outer core galactose and glucose residues. Typically, it also includes the outer core N-acetyl-D-
20   glucosamine residues. For example, it includes the outer core structure of Rb and typically also the structure of Ra, as shown in Fig. 2. It does not include the O-polysaccharide outer region (also called O-polysaccharide side chain).
25   Thus, one aspect of the invention generally features a method of reducing the adverse effects of endotoxemia in a warm-blooded animal (a mammal, typically a human patient), by administering an effective amount of a composition comprising complete-core, rough,
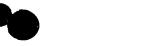30   lipopolysaccharide (LPS) antigen (e.g., an Ra LPS) of a Gram-negative bacterium, particularly E. coli K12. Preferably, the immunizing composition is a cocktail of complete-core, rough, lipopolysaccharide (LPS) antigen from other Gram-negative bacterium. Useful rough LPSs

- 8 -

are those from E. coli and Salmonella, particularly from
each of the five known chemotypes of E. coli: E. coli
R1, E. coli R2, E. coli R3, E. coli R4, and E. coli K12
(Jansson et al., Eur. J. Biochem. 115:571 (1981)). See
5 Fig. 3.   Only one core structure accounts for all known
Salmonella species, and any Ra Salmonella strain can be
used, for example Salmonella minnesota R60. Rietschel et
al. Infect. Dis. Clin. N. Am. 5:753 (1991). Complete
core LPS lacking polysaccharide side chains from other
10 Gram-negative bacteria that may be useful include those
from the family Enterobacteriaceae (i.e. the genera
Escherichia, Salmonella, Klebsiella, Citrobacter,
Shigella, Proteus, Edwardsiella, Enterobacter, Hafnia,
Serratia, Providencia, Morganella, Yersinia, Erwinia),
15 the family Pseudomonadaceae, e.g, Pseudomonas aeruginosa
and the family Bactoroides, e.g., B. fragilis. See,
generally, ESSENTIALS OF MEDICAL MICROBIOLOGY, 3'rd Ed., Volk,
et al., pp. 397 and 416 (J/P. Lippencott Co.
Philadelphia, PA (1986) for a compilation of Gram-
20 negative bacteria. The composition may include a
complete-core, rough, LPS antigen from several (two,
three, four or more) Gram-negative bacteria, each of
which is different (e.g., different species or at least
different strains of the same species) from the other.
25 In such mixtures, the core antigen from each of the four
bacteria may be present in functionally equal amounts
(e.g., in amounts which are intended to maximize the
expression of the common core epitope(s)).
          Desirably, vaccines should cause the patient to
30 produce an antibody that binds to an epitope in the core
region of the LPS core of at least one Gram-negative
bacterial strain whose LPS is not part of the
composition, thereby providing for cross-reactivity and
cross-protection. It is difficult to achieve genuine
35 vertical and genuine horizontal cross-reactivity and

cross-protection against smooth and rough gram negative
LPS, in particular in *E. coli*, the species most commonly
isolated from surgical and intensive care unit patients.
Cross-reactivity is of two kinds, which may be described
5   as horizontal and vertical. Vertical cross-reactivity
refers to an antibody's reaction with LPS's within the
same strain that are different sizes, i.e., having
different degress of substitution or length of the O-
specific side chain. Horizontal cross-reactivity refers
10 to an antibody's reaction with core structures that are
different -- i.e., different strains, species, etc. In
particular, the patient's antibody response desirably
will bind and protect against smooth as well as rough
forms of LPS. Without wishing to bind ourselves to any
15 particular theory, we believe that the epitope of the
immunogen used in the vaccine according to the invention
is accessible in both smooth and rough forms of LPS.

        It may be particularly useful to include the
antigen in a liposome structure. For example, the ratio
20 (weight:weight)of lipid in the liposome to the LPS
antigen is between 1:1 and 5000:1 (more typically between
10:1 and 1000:1). The liposome may include a component
to provide stability or alter the compound's charge,
selected from the group consisting of: phospholipid,
25 cholesterol, positively charged compounds, negatively
charged compounds, amphipathic compounds. Multilamellar
type liposomes (MLV) in particular may be used. Small or
large unilamellar liposomes (SUVs and LUVs) also may be
used.
30   The composition may be administered
intramuscularly intravenously, subcutaneously,
intraperitonealy, via the respiratory tract, or via
gastrointestinal tract. The dose of antigen can be
readily determined by standard dosage trials which
35 correlate dosage with titre and/or protection. A

- 10 -

functional dosage may be between 0.01 ng and 1000 ng per
kilogram of patient body weight, but further optimization
may indicate that higher dosages (up to 100 μg/kg of body
weight) are desirable consistent with safety and avoiding
5   untoward side effects. IgM antibodies can provide
suitable protection, and, were the goal is generation of
IgM antibodies, the composition may be administered
sufficiently in advance to permit IgM antibodies to be
produced (at least 2 days more typically longer) prior to
10 potential endotoxin exposure. Also in that case, the
composition would not be administered so far in advance
that the IgM response deteriorates substantially -- e.g,
less than 14 days prior to exposure. The composition may
be administered in multiple doses, the first of which is
15 administered at least 2 days prior to potential endotoxin
exposure.

      Antigen in the composition may be present as part
of bacteria that have been killed e.g., by heat or
formaldehyde. Alternatively, the antigen may be separated
20 from the bacterium before formulation of the composition.
Alternatively the LPS antigen can be in the form of
purified LPS or complexed to an acceptable carrier.
Appelmelk et al., J. Immunol. Meth., 82:199 (1985).

      The antigen may be chemically detoxified. The
25 bacterium may be genetically engineered for various
reasons, e.g., to reduce toxicity. The composition may
also include an adjuvant, e.g., alum.

      The invention also features vaccine compositions
described above in connection with the method. Thus, the
30 vaccine is comprised of an effective amount of one or
more complete-core, rough, LPS of a Gram negative
bacteria. Upon administration to a warm-blooded animal
the compositions stimulate the synthesis of antibodies
which recognize an epitope in the core region of the LPS
35 molecule and which are cross-protective against

endotoxemia caused by at least two different Gram-
negative bacterial strains having different core
structures. In particular, the antibodies synthesized in
response to the vaccine are cross-protective against

5 smooth LPS as well as complete core rough LPS (lacking O-
polysaccharide side chains). In E. coli, the antibodies
induced by the vaccine preferably react with all common
smooth strain isolates, and preferably also with rough
forms of all five core types (R1, R2, R3, R4, and K12).

10 Preferably the antibodies induced by the vaccine are also
reactive with both smooth and rough forms of LPS of
different strains of Salmonella.

   Furthermore, the vaccine described in this
invention preferably causes no unacceptable toxicity

15 following its administration to mammals. Toxicity may be
controlled by incorporating the LPS into liposomes, by
detoxifying the LPSs lipid A component and/or by
alteration of the lipid A component by genetic
manipulation of the above mentioned bacterial strains.

20   The vaccine composition can be used to immunize a
donor, from whom antibodies are harvested for
administration to a patient. Preferably the antibodies
harvested comprise a substantial percentage of IgM class
antibody.

25   Another aspect of the invention features a method
of quantitating lipopolysaccharide incorporated into
liposomes (PAS method). This method, unlike e.g., typical
radiolabelling methods, does not require conversion of
the lipopolysaccharide to a form which is unsuitable for

30 clinical use.

   Other features and embodiments of the invention
will be apparent from the following description of
specific embodiments.

- 12 -

Brief Description of the Drawings

Fig. 1 is a diagrammatic representation of smooth and rough LPS.

Fig. 2 is a diagrammatic representation of the
5 chemical structure of Salmonella R-mutants.

Fig. 3 is a diagrammatic representation of the chemical structure of known core types found in Salmonella and Escherichia complete core rough LPS.

Description of the Preferred Embodiments
10 Medical Indications

The patients to be treated with the vaccine include those at risk for endotoxin exposure. Specific candidates for active immunization include patients scheduled for surgery, patients subjected to chemotherapy
15 or radiation therapy as well as burn patients, trauma patients, dialysis patients and hospitalized (particularly ICU) patients, whether or not they exhibit sepsis or septic shock. Other potential candidates for vaccination include members of the military, fireman, and
20 policeman, as well as endurance athletes and livestock such as horses or cows.

LPS Component

As described above, the LPS antigen to be included in the vaccine can be any complete core LPS lacking O-
25 polysaccharide side chains, preferably from E. coli K12, as described below. Although Rb chemotypes may be used, the preferred embodiment is Ra or complete core chemotypes. LPS can be purified from cultured bacteria or purchased commercially, e.g., from Difco, Sigma, List
30 Biologicals, in Campbell, California. The organisms in question are widely available from depositories, including the National Culture Type Collection in England (NCTC); the University of Edinburgh collection in

- 13 -

Edinburgh Scotland,[1] and the Forschungsinstitut in
Borstell (FB), Germany D-2061.   Examples of specific
bacterial include, but are not limited to, the following
strains: E. coli K12 -- e.g., Edinburgh #MPRL2320; FB
5   W3100 or List Biologicals; E. coli R1 -- e.g.Edinburgh
#MPRL2316 or FB F470;   E. coli R2 -- e.g., Edinburgh
#MPRL2317 or FB F576;   E. coli R3 -- e.g., Edinburgh
#MPRL2318 or FB F653; E. coli R4 -- e.g., Edinburgh
#MPRL2431 or FB F2513; Ra S. minnesota R60 Edinburgh
10 #MPRL1265 or List Biologicals;   S. typhimurium Ra (e.g.
TV119,1542), P. aeruginosa PAC611 (e.g, Edinburgh
#MPRL1091) and K. aerogenes M10B (e.g, Edinburgh
#MPRL0954), S. minnesota Rb chemotype (e.g. Edinburgh
#MPRL1091) R345); Bacteroides fragilis -- NCTC 9343; B.
15 vulgatis NCTC 10583; B. thetaiotaomicron NCTC 10582.
        Without wishing to bind ourselves to a single
theory by which the invention operates, we note that the
inner core region may contain an important epitope in
terms of stimulating the synthesis of cross-reactive and
20 cross-protective anti-LPS antibodies.   However,
sufficient outer core structures may be necessary to
maintain the inner core epitope in a three-dimensional
structure which is similar to that encountered in
clinically significant LPS isolates (i.e. smooth and
25 rough forms of complete core LPS).   The absence of
polysaccharide side chain (i.e. rough LPS) allows the
core epitope to be the dominant epitope.   In smooth forms
of LPS, the polysaccharide side chain is a much more
dominant epitope than the core thus significantly
30 reducing the relative amount of anti-core antibody
produced.   In other words, vaccines containing smooth LPS
elicit primarily a serotype specific (i.e. anti-
polysaccharide side chain) antibody response as opposed

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[1]   University of Edinburgh Medical School (Edinburgh,
3Scotland), attention Ian Poxton, Ph.D.

- 14 -

to the anti-core response which is the focus of our invention.

E. coli K12 may be particularly useful because it is not generally present in the patient population. Therefore, K12 is less likely to provoke a memory response to the outer core, and more likely to provoke a cross-reactive memory response to the inner core.

If whole bacteria are to be included in the vaccine the bacterium will be killed by a technique well known to those in the art, such as heat killing or formaldehyde killing. In this case, the entire LPS of rough mutant bacterium will be included as part of the killed bacterium. It is desirable to avoid bacterial killing methods which can alter the core.

Alternatively, complete core LPS can be isolated from the desired bacteria according to standard techniques as outlined by Hancock et al., BACTERIAL CELL SURFACE TECHNIQUES, pp. 91 (John Wiley & Sons 1988). As noted, it is preferable to include all of the core LPS, without the O-polysaccharide outer LPS structures, i.e. use R-mutant bacteria expressing full LPS core.

Patient Response

As noted, the desired patient response is cross-protective antibodies that bind to the core of rough and smooth LPS of Gram-negative bacteria generally, regardless of whether their outer LPS structures are similar.

For example, in E. coli, the antibodies induced by this vaccine preferably react with all common smooth strain isolates, and preferably also with rough strain LPSs of all five core types (R1, R2, R3, R4, and K12). Preferably the antibodies induced by this vaccine are also reactive with different smooth and rough LPSs of Salmonella.

- 15 -

It is possible to achieve a vigorous and effective
antibody response using compositions with acceptable
levels of (or no) toxicity. The vaccine stimulates the
synthesis of antibodies which recognize an epitope in the
5   core region of the LPS molecule and which are cross-
protective against endotoxemia caused by at least two
different Gram-negative bacterial strains having
different LPS structures and in particular are cross-
protective against smooth strains as well as complete
10 core rough strains.

Typically, the vaccine will be a cocktail of the
purified LPS from different strains of bacteria,
preferably rough strains having a complete core, for
example a mixture of LPS from K12 with LPS from R1 and R3
15 rough strains of E. coli, or with the Ra strain of
Salmonella minnesota R60. E. coli R2 and R4 are less
important but also candidates. Preferred cocktails
(depending on the breadth of protection desired) include
K12 with R1; K12 with Pseudomonas (e.g., P. aeruginosa)
20 and Klebsiella (e.g., K. aerogenes). Since the
Bacteroides are a particularly significant population in
the gut, it may be important to protect specifically
against Bacteroides endotoxin by including Bactoeroides
in the cocktail. e.g., together with K12 or together with
25 K12, Pseudomonas and Klebsiella.

Alternatively, the purified LPS from one of these
strains, a mixture of any combination of these strains,
or a different strain of bacteria may be used in any
ratio of the individual strains in the case of use of
30 more than one LPS type.

The route of administration is preferably
subcutaneous or intramuscular, although any alternative
route which results in these immunogens reaching the
antigen presenting cells and antibody producing cells is
35 acceptable. Some other examples include but are not

- 16 -

limited to intravenous, intraperitoneal, and via the
respiratory or gastrointestinal tract.

    The dose of this composition should stimulate the
host to produce increased quantities of cross-reactive

5   and cross-protective antibodies levels, consistent with
avoiding toxicity, as described above.

    The composition is administered before endotoxin
exposure. To the extent that the vaccine works in part
by stimulating the host to synthesize antibodies of the

10 IgM class, the vaccine is preferably given between 2 to
14 days prior to potential endotoxin exposure.
Alternatively, additional doses of any of the possible
permutations of this vaccine may allow for greater
effectiveness and increases in desired antibody levels or

15 even further reduced toxicity. It is anticipated that in
most vaccinees the antibody response to inner core
determinants will be a secondary (i.e. memory) response
as opposed to a primary (i.e. naïve) response. This is
because most vaccinees will have been exposed at some

20 time in their lifetime to the LPS core epitopes,
presumably from LPS that has leaked through the gut
barrier into the bloodstream. In other words, an
important function of our method of vaccination is to
cause an increase in the serum concentration of

25 antibodies which may already be present, but at levels
which do not allow for sufficient protection from a toxic
exposure of LPS during periods of stress and trauma. The
above in no means suggests that there are not patients
who will also benefit from vaccination with this

30 invention by means of a primary (i.e. naïve) antibody
response.

    A vaccine with the LPS mentioned above is
preferably rendered non-pyrogenic and non-toxic by
incorporation of the LPS into liposomes. The liposome

35 (exclusive of the LPS component) may contain a

- 17 -

combination of (1) a phospholipid and cholesterol or (2)
a phospholipid, cholesterol and a negatively or
positively charged (lipophilic) amphipathic compound.
The phospholipid component may be selected from the group

5 comprising any lipid capable of forming liposomes,
including, but not limited to: any phosphatidyl-choline
derivative, glycerophosphatides, lysophosphatides,
sphingomyelins, and mixtures thereof. The negatively
charged (lipophilic) amphipathic compounds may be

10 selected from the group comprising di(alkyl)phosphates,
phosphatidic acid, phosphatidylserine,
phosphatidylglycerol, phosphatidylinositol, dicetyl
phosphate, or any other similar negatively charged
amphipathic compound that can impart a negative charge to

15 a liposome surface. When positively charged (lipophilic)
amphipathic compounds are employed; they are selected
from the group comprising alkyl amines, such as
stearylamine and hexadecylamine. The ratio in the
constituents of the liposomes (exclusive of the LPS) will

20 effect the liposomes' charge, rigidity, stability and may
vary greatly while still allowing for reduced toxicity
and increased immunogenicity of the LPS they contain.
Polyethylene glycol lipids (PEG) may be incorporated into
the liposomes, for example at approximately 10 to 20

25 mole%, in order to increase the amount of time that the
liposomes remain in the systemic circulation, thus
affecting their immunogenicity. Alternatively, very
rigid bilayers may be made by using lipids which are gel
phase at body temperature (37 degrees C), for example

30 distearoyl phosphatidylcholine or distearoyl
phosphatidylserine. The type of liposomes used is
preferably multilamellar liposomes (MLV) but
alternatively upon sonication, or by alternative methods
of manufacture, small or large unilamellar liposomes

35 (SUVs and LUVs) of varying sizes can be employed.

Different salt forms of LPS may alter the degree of incorporation of LPS into the liposomes, for example, the acid salt form, magnesium salt form, and calcium salt form may allow for increased incorporation due to their

5   increased hydrophobicity.

Liposomes are defined as closed vesicles, or sacs, which contain phospholipids (examples of which are lecithin and sphingomyelin) and which may contain other lipids (examples of which are cholesterol and other

10 steroids; charged lipids such as dicetyl phosphate and octadecylamine; glycolipids; fatty acids and other long-chain alkyl compounds; hydrophobic glycoproteins; and lipid soluble vitamins and lipoidal surfactant-like molecules). When shaken in the presence of an excess

15 amount of water, the lipid mixture is formed into discrete particles consisting of concentric spherical shells of lipid bilayer membranes which are referred to as multilamellar liposomes (MLV). Upon sonication, or by alternative methods of manufacture, small or large

20 unilamellar liposomes (SUV or LUV, respectively) can be formed.

Upon injection into animals and man, liposomes are taken up rapidly by cells of the reticuloendothelial system, particularly those of the liver. Because of the

25 relative impermeability of liposomes and their speedy removal from the circulatory system, substances such as lipid A and certain forms of LPS remain incorporated within the liposomes and are less likely to be exposed to cells and/or receptors through which they can exert

30 potentially toxic effects. Moreover, liposomes may allow for a prolonged effectiveness through slow biodegradation of the multilamellar membrane structure of the liposomes.

The toxicity of the lipid A component of the above mentioned complete core rough mutant strains also can be

35 reduced or eliminated by chemical detoxification as

- 19 -

described in Bhattacharjee A et al., WO 95/29662. The
preferred method for this detoxification maintains the
LPS configuration such that it still stimulates the
synthesis of antibody/ies which recognize an epitope in
5   the core region of the LPS molecule and which is cross-
protective against endotoxemia caused by at least two
different Gram-negative bacterial strains having
different core structures. In particular, the antibodies
synthesized in response to this vaccine are cross-
10 protective against smooth strains as well as complete
core strains. The detoxified LPS may be administered in
the form of purified LPS, or alternatively can be
incorporated into liposomes or complexed to an acceptable
carrier.
15   Alternatively the toxicity of the lipid A
component of the above mentioned strains of bacteria can
be reduced or eliminated by genetic alteration of the
bacterial strains as described in Somerville JE et al, J
Clin Invest 1996; 97:359-365. The resulting LPS from
20 these cells (in the form of heat killed cells) is reduced
in toxicity while still affording immunogenicity to LPS
core. The preferred method for this genetic alteration
maintains the LPS in a sufficient three-dimensional shape
that it still acts sufficiently as an immunogen in a host
25 to stimulate the synthesis of antibody/ies which
recognize an epitope in the core region of the LPS
molecule and which is cross-protective against
endotoxemia caused by at least two different Gram-
negative bacterial strains having different core
30 structures. In particular, the antibodies synthesized in
response to this vaccine are cross-protective against
smooth strains as well as complete core rough strains.
At the same time, this genetic process preferably renders
the LPS non-pyrogenic and non-toxic in the warm-blooded
35 animal. The LPS from these genetically altered bacterial

- 20 -

strains are preferably administered in form of purified
LPS incorporated into liposomes. LPS from these altered
strains can alternatively be administered in the form of
killed cells. Alternatively, the detoxified LPS may be
5 administered in the form of purified LPS, or
alternatively can be complexed to an acceptable carrier.

     Toxicity of any of the LPS rough antigen
compositions described in this invention may also be
reduced by other methods, for example, competitive
10 detoxification of lipid A by synthetic anti-endotoxin
peptides. Rustici et al. Science 259:361 (1993). An
alternative method of reducing toxicity is to administer
the LPS antigen with or at around the same time as an
anti-inflammatory agent, e.g., anti-TNF-alpha monoclonal
15 antibody. Fisher CJ et al. N Engl J Med 334:1697 (1996).

EXAMPLES
Examples herein offered to illustrate the invention are
not intended to limit the scope thereof. These examples
are offered to indicate experiments that may be done,
20 with no implication that all or any of the experiments
have in fact been performed.

Materials for liposome preparation
     Synthetic dimyristoyl phosphatidyl choline (DMPC),
dimyristoyl phosphatidyl serine, and dimyristoyl
25 phosphatidyl glycerol (DMPG) were purchased from Avanti
Polar Lipids. Cholesterol, 3-(N- Morpholino) propane
sulfonic acid (MOPS), periodic acid, and pararosaniline
based Schiff's reagent are purchased from Sigma. Sterile
saline and water for irrigation are purchased from Abbott
30 labs. 3M Empore solid phase extraction discs are
obtained from Fisher. Limulus amebocyte lysate (LAL)

- 21 -

standards and reagents are obtained from Associates of
Cape Cod.

     Materials and reagents are depyrogenated in one or
more of the following procedures: 1) Heating- glassware
5 are heated to approximately 180 degrees for no less than
16 hr; 2) Base Treatment- immersed in
isopropanol/concentrated potassium hydroxide for not less
than 2 hours; 3) Hydrogen Peroxide- immersed in
concentrated hydrogen peroxide for not less than 1 hour
10 at 70 degrees Celsius then rinsed with depyrogenated
water; 4) Ultrafiltration- solutions are filtered through
Amicon centriprep ultrafiltration membranes (3000 M.W.).

     All glassware, plasticware, solutions, and buffers
are free of contaminating endotoxin and verified by use
15 of the standard LAL assay (Pyrotell and Pyrochrome from
Associates of Cape Cod, Cape Cod, MA, USA).

     MOPS Saline Buffer and 0.5% periodic acid
solutions are stored at room temperature prior to use.
Lipid stock solutions are stored at -20 degrees Celsius.
20 LPS is stored dissolved in water for irrigation or in
0.1% TEA in glass or polystyrene containers (Evergreen)
at 4 degrees Celsius. Liposome preparations are stored
at 4 degrees Celsius. Pararosaniline based Schiff's
reagent is stored at 4 degrees Celsius prior to use.
25   Methods
Lipopolysaccharide

     Established strains of the following bacteria are
maintained according to standard procedures: K12, R1, R2,
R3, and R4 rough strains of E. coli, E. coli smooth
30 strains O18, O6, O157, O12, O15, and the Ra strain of
Salmonella minnesota R60 can be obtained as described
above. Smooth and rough LPS are purified according to the
established method described in Hancock et al., cited
above, pp 91-92. LPS from E. coli J5 (Rc chemotype), E.
35 coli, S. minnesota R595 (Re chemotype), and S. minnesota

- 22 -

(wild type), and S. typhimurium (wild type) can be
obtained from List Biological Laboratories Inc.
(Campbell, Calif). Native LPS may be used as well as
acid form (deionized) LPS made by electrodialyzing the
5   native LPS using the established method described in
Hancock et al. cited above at 93-95.

Incorporation of LPS into Liposomes

Liposomes are prepared by standard procedures.
Multilamellar vesicles (MLV) are prepared according to
10   the method of Dijkstra et al, J Immunol Methods 1988;
114:197-205, with some modifications, as indicated in
Methods #1 and #3, below, or a novel method (method #2).

For example, in method #1, 1 ml of 5 mg/ml of
native *E. coli* K12 (Ra) LPS in water was added to a 1 ml
15   aqueous dispersion of 50 mg lipid (DMPC:DMPG:cholesterol,
4:1:4, mol/mol), i.e. a lipid:LPS ratio of 10:1 (wt/wt).
The combined solution was then probe sonicated at 40-50
degrees Celsius for five 2 minute periods with 2 minute
wait periods in between each sonication. The solution
20   was then rotovapped to dryness and resuspended in a
buffer consisting of 4 mM MOPS, 153 mM NaCL, ph 7.8. LPS
not incorporated into the liposomes (free LPS) was
removed by centrifuging the preparation 3 times at 10,000
rpm in a Komp Spin KA 21.5 rotor for 10 minutes,
25   decanting all supernatant, and resuspending the pellet in
the original volume of buffer. This procedure was
repeated 3 times and reduced the concentration of
unincorporated LPS significantly. This method for
reducing free LPS can be used following most liposome
30   preparation methods.

In method #2, a solution of 5 ml
chloroform:methanol (2:1, v/v) was vortexed together with
2 ml of 0.1 M HCl. The lower organic phase was then
allowed to separate from the upper, removed, and used to

- 23 -

dissolve 5 mg of acid form LPS. 50 mg of lipid
(DMPC:DMPG:cholesterol, 4:1:4, mol/mol) was then disolved
in the LPS solution. The solution of codisolved LPS and
lipid was rotovapped to dryness, and resuspended as in
5 method #1.

In method #3, briefly, a 1 ml of a lipid stock
solution consisting of 4 mM dimyristoyl phosphatidyl
choline:1mM dimyristoyl phosphatidyl serine: 4 mM
cholesterol was rotovapped to dryness at 50 degrees
10 Celsius. 50 μl of a 0.1 mg/ml solution of mixed LPS
consisting of equal weights of LPS from Escherichia coli
strains K12, R1, R2, R3, R4 and Salmonella minnesota R60
suspended in 0.1% TEA is added with 150 μl of water. The
preparation was then vigorously vortexed and sonicated
15 for not less than 5 minutes in a bath type sonicator with
hot tap water in the bath. The preparations were then
rotovapped (or lyophilized) to dryness again and
resuspended in a buffer consisting of 4 mM MOPS 153 mM
saline pH 7.8 with vigorous vortexing.
20   In some of the methods of incorporation of LPS
into liposomes a single LPS type was used (e.g. 5 mg of
E. coli K12 (Ra)) while in others, equal amounts of
different LPSs were used (e.g. 0.83 mg of LPS from 6
complete core rough mutants).
25   Large unilamellar vesicles (LUV) were prepared
from MLV by repeated (minimum of 15) passages through a
depyrogenated pair of 100 nm polycarbonate membranes
housed in an Avestin Liposofast extruder which was also
depyrogenated prior to use by the hydrogen peroxide
30 treatment above.

LPS incorporated into liposomes from method #3 was
purified on a 1.1 x 28.5 cm BioGel A15M column using 4 mM
MOPS/153 mM saline as the running buffer and 0.7 ml/min
as the flow rate. This step maybe unnecessary since
35 there was no difference in the lymulate ameoba lysate

(LAL) activity between purified and unpurified liposomes due to the almost complete (>99.9%) incorporation of the free LPS into the liposomes.

In vitro Quantitation of Toxicity of LPS

5   Quantitation of the biological activity of the toxic lipid A component of all samples of vaccine and controls was accomplished using the standard LAL assay according to the manufacturer's instructions. (Pyrotell and Pyrochrome from Associates of Cape Cod, Cape Cod, MA,
10 USA). The rate of incorporation of free LPS into liposomes is generally reflected by a significant decrease of LAL activity following effective incorporation. Rates of incorporation of LPS into liposomes using traditional methods, including those
15 described above, usually exceed 90% and in some of the methods exceeded 99%.

Periodic Acid/Schiff's Base (PAS) Stain for LPS

Aliquots of LPS containing either MLV or LUV are diluted to 2 ml with water and extracted with 2 ml
20 toluene. After vortexing and a 5 minute 2800 rpm centrifugation in a Sorvall GLC-2 centrifuge, the upper toluene phase is removed and the aqueous phase is reextracted with 2 ml of fresh toluene. The aqueous phase and interphase are then taken, acidified with 30-50
25 μl of concentrated hydrochloric acid and extracted with 5 ml of chloroform:methanol (2:1). The aqueous phase is reextracted once with chloroform and the combined organic phases are dried under nitrogen at 50-60 degrees Celsius. Residues are then dissolved in chloroform:methanol (2:1)
30 and spotted on an Empore C8 extraction disc alongside an LPS standard curve spotted from 50% ethanol. Discs are dried in vacuo and incubated in 0.5% periodic acid at room temperature for approximately 30 minutes. They are then removed, rinsed with distilled water, and placed in

capped test tubes containing pararosaniline based
Schiff's reagent. The tubes are then warmed under hot
tap water until color develops. The discs are then
removed, rinsed again, and dried. Quantitation of the
5   resulting spots is done on an Agfa Arcus II desktop
scanner in conjunction with Adobe Photoshop and NIH Image
software.

                  Immunization and Pyrogenicity testing
             The pyrogenicity and toxicity of a vaccine
10   comprised of LPS is best measured in the rabbit model of
pyrogenicity established and outlined in the United
States Pharmacopiae.(USP 23, <151>, 1995, Rockville, MD)
In this established protocol, if an experimental
substance is administered and does not cause pyrogenicity
15   in rabbits relative to control animals, that substance is
defined as being non-pyrogenic and is unlikely to cause
fever or toxicity in other mammals, particularly humans,
following its administration. This protocol is an ideal
model since rabbits and humans are similarly sensitive to
20   endotoxin.
             Briefly, mature female New Zealand White rabbits
between 1.8 and 3.0 kg were sham tested twice to insure
their suitability for pyrogenicity testing. All testing
materials were administered as specified, either
25   intravenously (IV) as a 1 ml volume or intramuscularly
(IM) as a 0.6ml volume. Three rabbits are used per group
as necessary for tests completed to comply with
regulatory requirements. However, 2 rabbits per group
were sufficient to demonstrate large differences in
30   pyrogenicity between free LPS and LPS incorporated within
liposomes in examples described below. Temperatures of
the rabbits were measured to insure a steady baseline and
following administration of the test sample were
monitored at 15 minute intervals for 3 hours. A material

- 26 -

is considered non-pyrogenic if no rabbit shows an
individual rise in temperature of >0.5 degrees C above
baseline.

               Measures of Efficacy of Various Immunogens
5   A well recognized test for the effectiveness of an
immunogen is to administer the immunogen to a warm
blooded animal, typically a rabbit or a human subject,
and then withdraw blood samples periodically for the
determination of antibody levels. Blood samples were
10 drawn from a marginal ear vein into a red top tube the
day before testing and at different time points following
intramuscular (thigh muscle) immunization. Some
intramuscular immunizations were performed with the
administration of the adjuvant Alum. In these cases 0.3
15 ml(equal to 1.50 mg) of Alum (Alhydrogel 0.5% diluted
from stock 2%, Sergeant Co., Clifton, NJ) was mixed
thoroughly with 0.3 ml of vaccine immunogen prior to
administration. Blood was centrifuged, the serum
removed, and stored at -80 degrees C.

20   Binding of immunized rabbit serum to LPS by ELISA
               The cross-reactivity of sera from the immunized
rabbits was determined by a standard method of binding of
sera to purified LPS in enzyme-linked immunosorbent assay
(ELISA). Sera was obtained from rabbits before and after
25 immunization with different possible immunogens. This
sera was tested against numerous purified LPS in order to
determine the degree of horizontal cross-reactivity and
vertical cross-reactivity of the sera. The rabbit serum
following the method of active immunization described in
30 this invention bound to smooth and complete core rough
LPS of Escherichia coli as well as smooth and complete
core rough LPS of Salmonella. It demonstrated superior
binding than sera from rabbits immunized with immunogens

- 27 -

from prior art such as the extensively tested Rc J5
mutant of Escherichia coli, the Re mutant of Salmonella
minnesota, and lipid A.

LPS-polymyxin complexes

5   LPS molecular weights were calculated as described
previously (Scott BB et al., Serodiag Immunother Infect
Dis; 4:25(1990)). Purified LPS at 0.2mM in pyrogen-free
water (5ml) were mixed with polymyxin B sulphate (Sigma)
at 0.4mM in pyrogen free water (5ml), and sonicated
10 together with approximately ten short (5 second) bursts
of sonication. The resulting milky suspension was placed
in a 2000 MWCO membrane and dialysed overnight against
freshly-distilled water containing 0.05% Na axide (w/v)
to remove excess uncomplexed polymyxin B. The dialysed
15 material, often presenting as a flocular precipitate, was
recovered as a 10ml suspension with a presumptive LPS
concentration of 0.1 mM, and stored in polypropylene
Minisorb tubes (Nunc) at -40C.

LPS coating on microplates.

20   LPS-polymyxin complexes were resuspended with
sonication. LPS-polymyxin complexes were diluted 1:80 in
0.05 M carbonate-bicarbonate buffer pH 9.6, containing
0.05% sodium azide, which had been freshly prepared using
freshly-distilled water. The diluted complexes
25 (containing 1.25 micromolar LPS) were maintained in even
distribution in coating buffer by continuous rapid
stirring, and added at 100 microlitres per well to 96-
well microtitre plates or 8-well microtitre strips (in
96-well frames). The microtitre plates and strips used
30 were ELISA-grade polystyrene (Greiner, medium-binding
grade, flat-bottom wells): some other grades and some
other manufacturers microtitre plates may be unsuitable
for this assay. Plates were stacked, wrapped in plastic
(Clingfilm), and incubated overnight at 37C. The plates
35 were washed as previously described using phosphate-

- 28 -

buffered saline (PBS) with 0.05% (v/v) tween-20. A 5%
solution of bovine serum albumin (BSA) in PBS, containing
0.05% sodium azide, was added at 120 microlitres per
well. The plates were stacked, wrapped in plastic, and
5   incubated overnight at 37C. The plates were washed as
before, rinsed using freshly-distilled water, blotted by
inverting on absorbent paper, and dried at 37C. The
dried plates were sealed in plastic bags (one plate per
bag) and stored at -40C until used.

10   LPS ELISA

         Samples of test serum or plasma were diluted 1:200
in ELISA diluent [PBS / tween-20 (0.05% v/v) /
polyethylene-glycol 8000 (4% w/v) / BSA (1.0% w/v) /
sodium azide (0.05% w/v)], and added at 100 microlitres
15   per well, in triplicate, to LPS-coated plates. Plates
were incubated at 37C for 5 hours in a still-air (no fan)
incubator, then washed (PBS / tween). Other dilutions,
incubation durations, and test replication may be used,
however, for any given set of experimental control and
20   experimental groups should be subjected to identical
conditions. An alkaline-phosphatase-conjugated species-
specific, immunoglobulin heavy-chain-specific antibody
was used to determine the amount of each immunoglobulin
class bound. IgM antibodies were determined with mu-
25   chain specific conjugates and IgG antibodies were
determined with gamma-chain specific conjugates. Heavy-
chain specific species specific antibodies (e.g. anti-
rabbit Ig antibodies purchased from Harlan Sera-Lab (UK),
were used at 1:1000 in ELISA dilution buffer. The
30   diluted conjugates were added at 100 microlitres per
well, and plates were incubated for 120 minutes at 37C.
The plates were washed in PBS / tween, rinsed in
distilled water, blotted, and 100 microlitres of freshly-
prepared pNPP alkaline phosphatase substrate solution
35   (Sigma N-2770) was added per well. The color was allowed

to develop for 30 minutes at room temperature, and plates
were read (at 405nm and reference at 650nm) on an
automated ELISA plate reader (Molecular Devices Thermo-
max) and tests were expressed as the net optical density
5   at 405 nm and 650nm (reference). Alternatively, test
results can be read as above and expressed as a
percentage of a standard on the same plate using
automated curve fitting from device-related software
(Molecular Devices Softmax). Test samples can be
10 compared to a laboratory standard imuune serum from the
same species, placed as a triplicate series of 8 doubling
dilutions in ELISA diluent (standard curve) from, say,
1:50, down one column of triplicate wells on each
microplate.

15   <u>Binding of immunized rabbit serum to LPS by Western</u>
                        <u>Blotting</u>

        The cross-reactivity of sera from the immunized
rabbits was also determined by the standard method of
binding of sera to purified LPS in Western blotting.
20 Sera was obtained from rabbits before and after
immunization with different possible immunogens. This
sera was tested against numerous purified LPS in order to
determine the degree of horizontal cross-reactivity and
vertical cross-reactivity of the sera. The rabbit serum
25 following active immunization with the method of active
immunization described in this invention bound to smooth
and complete core rough LPS of Escherichia coli as well
as smooth and complete core rough LPS of Salmonella. It
demonstrated superior binding than sera from rabbits
30 immunized with immunogens from prior art such as the
extensively tested Rc J5 mutant of Escherichia coli and
is expected to demonstrate superior binding compared with
serum from rabbits immunized with purified lipid A as
well as rabbits immunized with LPS from the Re mutant of
35 Salmonella minnesota.

- 30 -

PAGE analysis was performed on 12% (w/v) acylamide
gels with the buffer system of Laemmli (Nature; 227:680-
85 (1990)), except SDS was omitted from the stacking and
separating gel buffers.   Samples of LPS (5-10 micrograms
5   for rough LPS and 20-25 micrograms for smooth LPS, mixed
with Laemmli's sample buffer) were loaded onto the gel
and electrophoresed at 60 volts until the sample had
entered the separating gel, and then at 150 volts until
the dye front had migrated 7.5 cm through the gel. The
10 separated LPS was stained by the modified silver stain of
Hancock and Poxton (Bacterial Cell Surface Techniques,
pub. Wiley, p. 281 (1988)) except that oxidation was done
for 15 minutes.   For immunoblotting, the LPS was
transferred to nitrocellulose membrane (Schleicher and
15 Schuell, Germany), 0.2 micrometer pore size at 10-12
volts for 16 hours at 4 degrees Celsius with the Tris,
glycine, methanol buffer of Towbin et al, Proc Natl Acad
Sci USA; 76:4350-54 (1979)).   The transferred LPS was
immunostained as described in Hancock and Poxton
20 (Bacterial Cell Surface Techniques, pub. Wiley, p. 204-5
(1988)), except that incubation times and serum dilutions
were selected to give best results, and the immunoblot
was rinsed prior to developing.

The LPS content extracted from a smooth bacterium
25 was separated by electrophoresis into bands corresponding
to LPS molecules having different molecular weights,
depending on the size of the O-specific side chain.
These LPS molecules ranged from LPS molecules without any
O-specific side chain (equivalent to the size of a
30 complete core (Ra) rough mutant) to LPS molecules having
40 or more units in the side chain.
Protection Based on Inhibition of LPS Induced Stimulation
of the LAL Assay by Serum from Vaccinated Rabbits
The limulus amebocyte lysate (LAL) assay is an
35 established test for the biologic activity/toxicity of

lipid A (the toxic component of LPS). This assay
quantitates the activity of a biologically active LPS,
whereas in the presence of protective anti-LPS antibodies
there is significant inhibition of the LAL test due to
5   competitive binding and neutralization of the LPS. There
are numerous methods for using the LAL assay to
demonstrate protection from anti-LPS antibodies. LAL
test kits can be purchased from manufacturers, e.g.
Coatest Endotoxin from Chromogenix, Sweden. Different
10 versions of the LAL assay can be used, e.g. gel-clot
version or chromogenic version.

In an example of this method, a kinetic
chromogenic LAL assay was used. In this assay, serum was
obtained from a rabbit before and after vaccination with
15 complete core LPS incorporated into liposomes. E.coli R1
LPS (1000 micrograms/ml) was diluted 1/5 into pyrogen-
free water on a microplate, then five-fold diluted across
the plate, allowing for a final volume of 40 ul in 5
wells (1000 ug/ml to 1.6 ug/ml). Dilutions of LPS are
20 performed to avoid: 1) having only wells in which there
is not enough LPS to cause LAL activation in the presence
of serum; 2) having only wells in which there is an
excessive amount of LPS. There was a dilution series for
each serum or control to be tested (total 3 rows).
25 Row A- pyrogen-free water
row B- day-0 rabbit serum (complete core immunogen)
Row C- day-63 rabbit serum (complete core immunogen)
20 ul of water or rabbit serum was added to each well and
left for 30 minutes at room temperature. 20 ul of
30 LAL/substrate was then added using a multi-tip pipettor
(start of reaction) to each well, placed immediately in
reader, and read at 20sec intervals for 120 minutes. The
time (seconds) from start of reaction to an optical
density (OD) of 0.5 is a reflection of the degree/speed
35 of LAL activation. In the presence of water and LPS, the

- 32 -

LAL reaction proceeded quickly and an OD of 0.5 was
reached quickly. Pre-immunization serum contains some
anti-LPS antibodies as well as non-specific inhibitors of
LPS (e.g. lipoproteins). This serum partially
5   neutralized the LPS thus slowing down the LAL reaction
and increasing the time required for the OD to reach 0.5.
Post-immunization serum from the complete-core immunized
rabbit resulted in a significant
neutralization/protection of the R1 LPS as evidenced by
10 the marked prolongation of the time required to reach an
OD of 0.5. Similar results were obtained using other
types of stimulating LPSs and were consistent with the
ELISA LPS binding data described earlier.

        In another method using inhibition of LAL
15 activity, a known quantity of LPS is added to several
dilutions of serum from both pre- and post-vaccinated
subjects and the LAL activity (EU) for each is compared.
Protective anti-LPS antibodies in serum, in particular
the post-vaccination serum, result in lower LAL activity
20 compared with the pre-immunization value.

Cross-Protection Based on Inhibition of LPS-Induced IL-6
Secretion by Murine Peritoneal Macrophages

        Several monokines including tumor necrosis factor
(TNF), IL-1, and IL-6 mediate many of the
25 pathophysiologic events associated with Gram-negative
endotoxemia. These monokines are secreted by monocytes
and macrophages both in vitro and in vivo, in response to
LPS. Serum with protective anti-LPS antibodies block the
LPS induced macrophage or monocyte stimulation as shown
30 in the following assay. This type of assay can be done
using established mouse cell lines (e.g. J774.2),
established human cell lines (e.g. THP-1), freshly
obtained mouse peritoneal cells (C3H/HeN), or freshly
obtained human monocytes/macrophages. Following
35 stimulation with LPS, the assay can test for TNF or IL-6

- 33 -

levels. The amount of any type of LPS (e.g. E. coli R1)
used to stimulate the cells needs to be determined in
preliminary experiments. Too little LPS results in
inadequate stimulation and undetectable monokine levels
5   in the control group whereas too much LPS can overwhelm
even a large amount of protective antibody. Serum with
protective antibodies results in a lower monokine level
(for example, the TNF level), compared with serum
controls following stimulation with LPS.

10   In one example, this assay was performed as in
Delahooke DM et al. Infection and Immunity, 1995, p 840-
46. This assay uses a human cell line (THP1) which
secretes TNF following stimulation with LPS. In this
assay, the serum after vaccination with complete core
15   antigen demonstrated significant inhibition of TNF
induction

In another example, mouse peritoneal cells
(C3H/HeN) are obtained by peritoneal lavage with 0.34 M
sucrose in distilled water. Peritoneal cells are seeded
20   at 5×10^5 cells/ml in 0.2 ml serum free medium (IMDM-ATL,
Schreier and Tees, Immunological Methods, Vol. II, Acad.
Press (1981) : 263) and cultured for 4 hours at 37
degrees C in the presence or absence of (1) LPS, e.g. LPS
from E. coli R3 (0.05 ng/ml) or E. coli O18 (0.05 ng/ml)
25   or S. minnesota wild type smooth (0.05 ng/ml) or S.
minnesota R60 (0.05 ng/ml); and (2) in the presence or
absence of diluted or undiluted serum from rabbits
immunized with varying immunogens, e.g. composition
described in this invention or Rc J5 mutant of E. coli or
30   Re mutant of S. minnesota. The supernatants are
recovered and the amount of IL-6 present in the
supernatants is then measured using the IL-6 dependent
hybridoma cell-line B13.29 (Aarden et al., Eur. J.
Immunol. 1987, 17, 1911) as follows:

- 34 -

B13.29 cells are seeded at 2.5 x 10^4 cells/ml in serum free medium and cultured for 72 hrs in the absence of IL-6 and in the presence or absence of culture supernatant. Aliquots of the cultures (200 μl/well) are
5 distributed in flat bottomed microtiter plates. IL-6 concentration in the supernatants is calculated in relation to a standard curve of IL-6. The post-immune serum should cause reduced IL-6 secretion compared to the pre-immune serum.

10   Cross-Protection from Lethal Dose of Endotoxin
         Another measure of the effectiveness of this invention is its ability to confer cross-protection against LPS. A mouse lethality model is used in which mice are immunized intraperitonealy on Day 0 with either
15 the invention, appropriate controls, or immunogens described previously such as the Rc J5 mutant LPS of Escherichia coli and the Re mutant LPS of Salmonella minnesota. A second dose of antigen is administered on Day 7 and Day 14. Between days 19 and 21 endotoxin can
20 be administered intravenously in a 95% lethal dose of a particular LPS to groups of six female C57BL/6 mice, 6-8 weeks old. Galactosamine (D-GalN) (800 mg/kg) is administered intraperitoneally at the time of the LPS. The minimum intravenous dose of LPS required to kill
25 approximately 95% of the animals (LD_{95}) is determined in preliminary experiments. Survival is recorded up to 24 hours.   Alternative methods can be used in this protocol without substantially changing its ability to demonstrate whether immunization with a particular
30 immunogen results in protective antibodies. For example, other strains of mice can be suitable, the galactosamine dose can be modified, and the dosing schedule of vaccination can be altered so as to administer more doses. The experiment can also be performed by isolating

serum from rabbits immunized with the experimental
immunogen, and then administering the serum to mice prior
to challenge with galactosamine and LPS. This method
using passive immunization can be used to demonstrate the
5 protective nature of serum.

Example 1

Liposomes containing *E. coli* K12 complete-core LPS
were made according to method #2, above. Liposomes
containing a cocktail of six complete-core LPS (*E. coli*
10 R1-R4, K12, S. minnesota R60 Ra) were made according to
the method described above.

Groups of three mature rabbits were immunized
intramuscularly using a dose of 0.5mg of antigen with
Alum (as described earlier) on days 0, 14, and 56. On
15 days 0, 14, 21, 56, and 63, blood was withdrawn and
processed as described earlier.

Using the ELISA method described earlier, rabbits
immunized with K12 demonstrated increases in both IgM and
IgG antibody levels to smooth and rough forms of LPS from
20 E. coli and Salmonella bacteria.

Using the Western/immunoblot method described
earlier, serum from both groups of immunized rabbits
demonstrated enhanced binding to smooth and rough forms
of LPS from E. coli and Salmonella typhimurium. Binding
25 of serum from rabbits immunized to K12 alone to smooth
forms of LPS from E. coli (serotypes 018, 012 and 015) as
well as to the LPS from Salmonella typhimurium wild type
was comparable to binding of serum from rabbits immunized
with the cocktail of six complete LPS cores.

30   ### Example 2

Purified lipopolysaccharide in equivalent amounts
from the following rough strains of bacteria having a
complete core, R1, R2, R3, R4, and K12 strains of Ra E.
coli and the Ra strain of Salmonella minnesota R60, are
35 tested either alone or following their incorporation into

- 36 -

liposomes as described earlier. Heat killed cells and
liposomes alone are also evaluated. Compositions are
made according to the materials and methods described
earlier.

5 <u>Groups</u>

1.   100 μg of MLV (no LPS)

2.   Purified LPS (3 ng total) from E. coli R1

3.   purified LPS (0.3 ng total) (0.05 ng each of E.
coli K12,R1,R2,R3, and R4, and S. minnesota R60)

10 4.   purified LPS (3 ng total) (0.5 ng each of E. coli
K12,R1,R2,R3, and R4, and S. minnesota R60)

5.   purified LPS (30 ng total) (5 ng each of E. coli
K12,R1,R2,R3, and R4, and S. minnesota R60

6.   purified LPS (300 ng total) (50 ng each of E. coli

15 K12,R1,R2,R3, and R4, and S. minnesota R60)

7.   Cocktail of the 6 LPSs mentioned above
incorporated into MLVs as 1:1000 ratio by weight
(LPS:lipid) (3 ng total) (0.5 ng each of E. coli
K12,R1,R2,R3, and R4, and S. minnesota R60)

20 8.   Cocktail of the 6 LPSs mentioned above
incorporated into MLVs as 1:1000 ratio by weight
(LPS:lipid) (30 ng total) (5 ng each of E. coli
K12,R1,R2,R3, and R4, and S. minnesota R60)

9.   Cocktail of the 6 LPSs mentioned above

25 incorporated into MLVs as 1:1000 ratio by weight
(LPS:lipid) (300 ng total) (50 ng each of E. coli
K12,R1,R2,R3, and R4, and S. minnesota R60)

10.   Cocktail of the 6 LPSs mentioned above
incorporated into LUVs as 1:1000 ratio by weight

30 (LPS:lipid) (3 ng total) (0.5 ng each of E. coli
K12,R1,R2,R3, and R4, and S. minnesota R60)

11.   Cocktail of the 6 LPSs mentioned above
incorporated into LUVs as 1:1000 ratio by weight

(LPS:lipid) (30 ng total) (5 ng each of E. coli
K12,R1,R2,R3, and R4, and S. minnesota R60)

12.   Cocktail of the 6 LPSs mentioned above
incorporated into LUVs as 1:1000 ratio by weight
(LPS:lipid) (300 ng total) (50 ng each of E. coli
K12,R1,R2,R3, and R4, and S. minnesota R60)

13.   Heat killed cells from E. coli R1 (amount that
has same LAL activity as 3 ng of E. coli R1 LPS)